Articles

Resistance of melanized feathers to bacterial degradation: is it really so black and white?

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Melanins are common feather pigments that contribute to signaling and crypsis. Melanins may also help feathers resist feather-degrading bacteria (FDB). Two recent studies (Goldstein et al. 2004, Grande et al. 2004) tested the resistance of melanized versus unmelanized feathers to FDB using *in vitro* experiments, but draw opposite conclusions. Goldstein et al. (2004) concluded that melanized feathers resist FDB more than unmelanized feathers, while Grande et al. (2004) concluded that unmelanized feathers resist FDB more than melanized feathers. To resolve this conflict in the literature, we replicated previous studies but included additional tests not previously used. We inoculated melanized and unmelanized feathers of domestic geese *Anser anser domesticus*, with the FDB *Bacillus licheniformis* and measured bacterial activity every two days over two weeks. Three metrics of bacterial activity on feathers were measured: soluble protein content around feathers in solution, bacterial growth on feathers, and loss of feather mass. The latter two metrics were not considered in the aforementioned studies, which indirectly measured bacterial activity. We conducted two trials, one in which feathers were sterilized by autoclaving before inoculation (Goldstein et al. 2004, Grande et al. 2004), and a second in which feathers were sterilized by ethylene oxide gas. This allowed us to test whether autoclaving, done in previous studies, influences bacterial activity on feathers and could confound results. In both trials, unmelanized feathers degraded earlier, supported greater bacterial growth, and lost more mass than melanized feathers. These results support the findings of Goldstein et al. (2004); melanized feathers are more resistant to FDB than unmelanized feathers. Thus, using direct metrics of bacterial activity, we resolve a current conflict in the literature. We also found that autoclaving feathers influences FDB activity on them, and thus autoclaving should be avoided in future studies.

Melanins are the most common pigments of feathers and are responsible for the majority of black, brown, and earth-toned colours of avian plumage (McGraw 2006). The putative functions of melanized feathers are many, ranging from thermoregulation (Walsberg 1983) to crypsis (Zink and Remsen 1986) to social signaling (Griffith et al. 2006). Placement of melanins into feathers appears to be costly and subject to environmental influences, much like carotenoid pigmentation (Griffith et al. 2006, also reviewed in McGraw 2006). Variation in feather melanization may influence feather condition, as melanized feathers are harder and more resistant to physical abrasion than unmelanized (white) feathers (Bonser 1995, Burtt 1979, 1986). Feather wear can influence flight performance (Swaddle and Witter 1997, Williams and Swaddle 2003) and thermoregulatory ability (Booth et al. 1993), and thus melanization could reduce costs associated with feather wear.

The physical properties of melanized feathers compared to unmelanized feathers have led to the suggestion that melanized feathers are resistant to feather-feeding ectoparasites such as lice, and there is evidence consistent with this hypothesis (Kose et al. 1999, but see Bush et al. 2006). Similarly, melanized feathers may be more resistant than unmelanized feathers to damage by feather-degrading bacteria (FDB) (Goldstein et al. 2004, but see Grande et al. 2004). FDB are a polyphyletic assemblage of microbes that produce proteolytic keratinase enzymes that hydrolyze β-keratin (Gupta and Ramnani 2006, Onifade et al. 1998), the primary constituent of feathers. FDB occur within the plumage of several birds (Burtt and Ichida 1999, Whitaker et al. 2005), and, for some species at least, are a ubiquitous feature of plumage (e.g. FDB detected on 66/67 (99%) of eastern bluebirds *Sialia sialis*, in southern Virginia, U.S.A.; A.R. Gunderson unpubl. data). Despite the potential for FDB to be a potent selective force shaping feather evolution (Burtt and Ichida 1999, Clayton 1999), little is known about how these microbial symbionts function on live birds. FDB were first isolated from wild bird feathers less than 10 years ago (Burtt and Ichida 1999), and in the short time since only a handful of studies have addressed interactions between birds and FDB (e.g. Burtt and Ichida 2004, Cristol et al.

2005, Shawkey et al. 2007, Shawkey et al. 2003, reviewed in Gunderson 2008).

To date, two studies (Goldstein et al. 2004, Grande et al. 2004) have tested the hypothesis that melanized feathers are more resistant to FDB than unmelanized (white) feathers; however, these studies reached opposing conclusions. Goldstein et al. (2004) suspended unmelanized (white) and melanized (black) secondary flight feathers of domestic chickens *Gallus gallus,* in a buffer media (referred to as "feather solution") and inoculated them with the FDB *Bacillus licheniformis.* Feather degradation rates were determined by measuring the soluble protein concentration in feather solution over the course of six days. This method assumes that proteins in feather solution are the result of bacterial activity on feathers. Feather solution had higher soluble protein content with unmelanized than melanized feathers (Goldstein et al. 2004), suggesting feather melanization inhibits *B. licheniformis.* On the contrary, Grande et al. (2004) found that melanized feathers degrade more quickly than unmelanized feathers. In their study, melanized and unmelanized feathers from two (*Ciconia ciconia, Corvus corax*) and three (*Eudocimus ruber, Egretta garzetta, Ciconia ciconia*) species, respectively, were placed in feather solution and inoculated with *B. licheniformis.* Feather degradation was scored subjectively by observing each flask and visually estimating feather damage. Melanized feathers showed signs of degradation before unmelanized feathers, and melanized feathers were more damaged than unmelanized feathers at the conclusion of the experiment (Grande et al. 2004). This study also included feathers coloured with carotenoids, which also appeared to be more resistant to bacterial degradation than melanized feathers (Grande et al. 2004).

The results of these two studies have received considerable attention (see Bortolotti 2006, McGraw 2006, Shawkey and Hill 2004) because they suggest feather pigmentation may play a role in the resistance of feathers to parasites. Thus, those studying avian colouration may have overlooked an important mechanism that modulates colour expression. The question remains, however: do melanins impart bacterial resistance to feathers or do they make feathers more susceptible to bacterial degradation? Based on the two studies discussed above, there is no clear answer to this question. Furthermore, we felt there were aspects of the Goldstein et al (2004) and Grande et al. (2004) studies that suggested their results be regarded cautiously. Goldstein et al. (2004) do not present data on replicate experimental units for each feather type. Their results include no measures of error and were not subjected to statistical scrutiny. Furthermore, the authors included an uninoculated control flask for unmelanized feathers only, but not melanized feathers. This omission of a control treatment could confound their results because, as the authors state themselves and as is discussed above, melanized feathers are more resistant to physical damage than unmelanized feathers. Thus, the discrepancy in degradation rates could have been the result of their initial feather sterilization method (autoclaving at 121° C for 15 min) and/or their incubation conditions (agitation at 120 rpm at 37° C) weakening unmelanized feathers more than melanized feathers, causing them to be less resistant to bacterial degradation. Interpretation of the Goldstein et al. (2004) data is further complicated by the fact that they do not present or discuss the results for the unmelanized control that

they prepared. Grande et al. (2004) did have replication in their study; however, they determined feather degradation rates subjectively on a scale of 1–5 based upon visual inspection of the feathers in the flasks, using a criterion determined *a posteriori* (Grande et al. 2004). Grande et al. (2004) also did not have controls for all bird species used in their study and did not present data on controls.

In this study, we investigated whether melanized feathers are more resistant to bacterial degradation than unmelanized feathers, while addressing our concerns about the studies discussed above. Because of the physical characteristics of melanized feathers, we hypothesized that melanized feathers would be more resistant to FDB than unmelanized feathers. We thus predicted that FDB would degrade melanized feathers more slowly than unmelanized feathers. To test this, we quantified peptides released into solution around inoculated feathers (Goldstein et al. 2004), as well as bacterial growth and loss of feather mass (neither of which were quantified in the aforementioned studies).

Before feather degradation trials can be conducted, feathers must be sterilized to ensure that bacteria initially present on the feathers do not contribute to feather degradation or alter the activity of bacterial inocula. Feather degradation trials were conducted using two different feather sterilization methods, autoclaving and ethylene oxide gas. Goldstein et al. (2004) and Grande et al. (2004) both sterilized feathers by autoclaving them at high temperature and pressure. High temperature and pressure conditions can influence the structure of feathers, and such conditions are often used to pre-treat feathers when they are being processed into agricultural foodstuffs (Kim and Patterson 2000). Thus, autoclaving may make feathers more readily degradable, leading to ecologically questionable results in bacterial feather degradation trials. Furthermore, because melanized feathers are thought to be more robust than unmelanized feathers, autoclaving may differentially influence feather structure depending on pigmentation, which would confound the experiment. Specifically, melanized feather structure might be less affected by autoclaving than unmelanized feathers. Ethylene oxide gas sterilization does not expose feathers to high temperature and pressure conditions (see Materials and methods), and thus should not influence feather structure.

Materials and methods

We used unmelanized (white) and melanized (dark brown) retrices of domestic geese *Anser anser domesticus,* obtained from a commercial breeder. In each degradation trial, 300 mg of feathers (consisting of several small feather pieces from different feathers and, probably, different individuals) was placed in each of twelve 125 ml Erlenmeyer flasks; six flasks contained unmelanized feathers and six flasks contained melanized feathers. We used only the distal (approximately) 10 cm of feathers to ensure that most of the feather mass consisted of barbs and barbules. The rachis constitutes most of the mass of the proximal region and is not usually degraded by *B. licheniformis* (Ramnani et al. 2005).

We conducted degradation trials with feathers sterilized with autoclaving or ethylene oxide gas. In trials in which

feathers were sterilized by autoclaving, feathers were placed dry in flasks and autoclaved for 15 min at 121° C (unmelanized feathers) (Goldstein et al. 2004) or 18 min at 121° (melanized feathers). Melanized feathers had to be autoclaved for 18 min, as 15 min of autoclaving repeatedly failed to sterilize flasks containing melanized feathers ($N = 30$ flasks). After autoclaving, 50 ml of sterile phosphate buffered saline (PBS), pH $= 7.25$, was added to each flask. We did not autoclave the feathers in PBS because in preliminary trials this appeared to contribute to feather dissolution. For ethylene oxide gas sterilization, feathers were incubated in a chamber at 54° C with 170 g of ethylene oxide gas, followed by 12 hours of aeration. Feathers were sterilized dry and then placed in flasks containing 50 ml sterile PBS. For both sterilization methods, flasks were kept at 35° C for 24 h after the addition of PBS to allow soluble proteins associated with the feathers to enter solution and to allow any potential contaminants to grow. We then sampled the feather media to take an initial reading of soluble protein concentration before experimental bacterial inoculation. From these samples, we plated 20 μl of the solution on trypticase soy agar (TSA), a general growth medium, to ensure that the flasks were not contaminated. Three flasks of unmelanized feathers and three of melanized feathers were then inoculated with FDB, resulting in a total of three treatment (inoculated with bacteria) and three control (not inoculated with bacteria) replicate flasks per feather pigment type, per trial.

Treatment flasks were inoculated with pure cultures of *B. licheniformis* strain OWU 138B, a FDB originally isolated from the plumage of a willow flycatcher, *Empidonax traillii* (Goldstein et al. 2004). *B. licheniformis* is a well-studied FDB (e.g. Burtt and Ichida 1999, Ichida et al. 2001, Shawkey et al. 2003, Whitaker et al. 2005) that appears to be common within the plumage of many species (Burtt and Ichida 1999, Whitaker et al. 2005). Approximately 500,000 cells of *B. licheniformis* were administered to each treatment flask. To accomplish this, the FDB were streaked on TSA 24 hours before feathers were inoculated, and were incubated at 35° C. Pure isolated colonies were removed from the plates with a sterile cotton swab and used to inoculate a solution of sterile PBS. The concentration of bacteria in the solution was determined by measuring the optical density at 600 nm with a Bio-Rad Smart Spec 3000 (Bio-Rad), and this concentration was used to calculate the volume required to inoculate the flasks with the target number of bacteria. Among trials the volume of bacterial solution added to treatment flasks ranged from 1–4 μl, and, thus, did not significantly affect the volume of solution in treatment flasks. After inoculation, all flasks were placed in a 37° C incubator with agitation at 120 rpm.

Protein sampling and concentration assay

Every 48 h, over a 12-d period, we withdrew 500 μl aliquots of feather solution from each flask and all aliquots were stored at −20° C. Upon thawing for protein concentration assay, samples were centrifuged at 10,000 g for 5 min to remove particulate feather matter and bacteria from the supernatant. The soluble protein content of each sample was determined by measuring the absorbance of the supernatant at 280 nm (Lucas et al. 2003) using a

Nanodrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE). This method assumes that proteins in solution are derived from bacterial feather degradation, but does not specifically measure the concentration of oligopeptides produced by keratin cleavage. Other proteins present in feathers or produced by bacteria most likely contribute to the soluble protein content as well.

Viable cell counts

Bacteria were quantified every 48 h, at the time of protein sampling, using the plate count method (Hattori 1982). For each flask, 20 μl of feather solution were plated on TSA from the following dilutions: 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Plates were incubated at 35° C for 24 h, after which all colonies were counted. Plate counting also allowed us to determine if flasks had become contaminated with bacteria other than *B. licheniformis* over the course of the trials. No contamination was detected.

Change in feather mass

Before sterilization, the feathers to be placed in each flask were dried for 48 h at 55° C and weighed to the nearest 0.1 mg on an analytical balance (Ohaus AS60). After trials, the feathers were again dried, this time for 72 h (we found that this increase in drying time was necessary to remove all moisture after the feathers had been suspended in aqueous solution during trials) and weighed to the nearest 0.1 mg.

Data analysis

We used repeated measures analysis of variance (ANOVA) for all analyses unless otherwise noted. Analyses performed on bacterial growth data were log transformed to improve normality and reduce disparities in variance among groups. All statistical tests were performed using the R statistical programming package (v. 2.4.1) employing two-tailed tests of probability.

Results

Autoclave sterilization trial

Feather media significantly increased in soluble protein content for inoculated unpigmented (time by treatment interaction, $F_{7,28} = 10.20$, $P < 0.001$; Fig. 1A) and melanized (time by treatment interaction, $F_{7,28} = 2.87$, $P = 0.02$; Fig. 1B) feathers relative to controls. However, we did not detect a degradation difference between inoculated melanized and unmelanized feathers (time by pigment by treatment interaction, $F_{7,56} = 0.59$, $P = 0.764$). As a coarse metric of when degradation began for feathers of each pigmentation type, we used the time at which the 95% confidence interval of the mean of neither treatments nor controls included the mean of the other group. Initial protein content at the beginning of the experiment (i.e., time $= 0$) was standardized to zero for each flask by subtracting each flask's initial protein concentration from all subsequent measurements. Using this criterion,
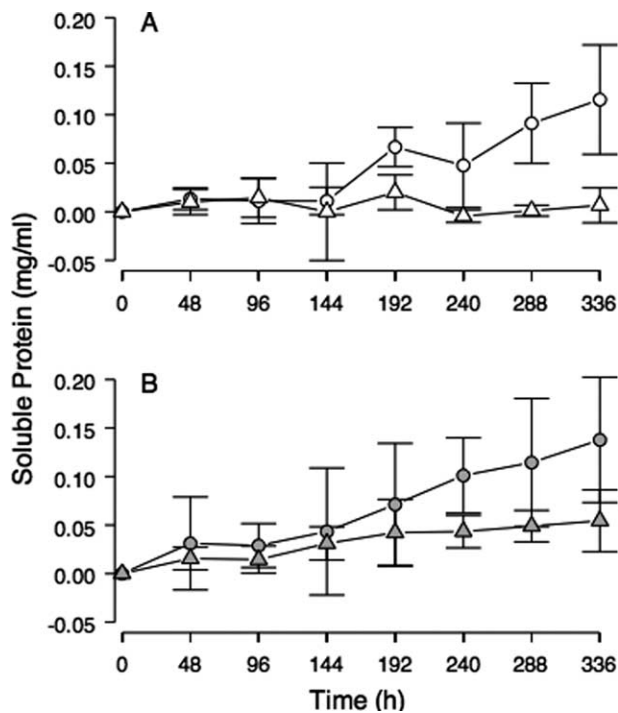
Figure 1. Mean soluble protein content of media surrounding autoclaved domestic goose feathers inoculated with *B. licheniformis*. (A) Unmelanized feathers. (B) Melanized feathers. *Circles*, feathers inoculated with *B. licheniformis*; *triangles*, uninoculated controls. Bars indicate 95% confidence intervals.

unmelanized feathers demonstrated clear signs of degradation after 192 hours (Fig. 1A), while melanized feathers showed clear signs of degradation after 240 hours (Fig. 1B).

B. licheniformis grew to higher densities on unmelanized feathers than on melanized feathers (time by pigment interaction, $F_{5,20} = 9.72$, $P < 0.001$; Fig. 2A). Plate counts from 192 h were removed from this analysis because the counts were performed incorrectly, as were plate counts from 336 hours because one flask could not be counted, which led to statistical imbalance. Inoculated unmelanized feathers lost more mass than unmelanized controls (time by treatment interaction, $F_{1,4} = 23.49$, $P = 0.008$). Similarly, inoculated melanized feathers trended towards more mass loss than control melanized feathers (time by treatment interaction, $F_{1,2} = 17.31$, $P = 0.053$; Fig. 2B). One melanized control flask and one melanized treatment flask had to be removed from the mass data set because an error occurred in their measurement, so we were unable to statistically compare feather mass loss across pigments due to statistical imbalance. However, on average, unmelanized feathers lost an order of magnitude more mass than melanized feathers.

Ethylene oxide sterilization trial

Soluble protein content around feathers inoculated with bacteria increased significantly relative to controls for unmelanized feathers (time by treatment interaction, $F_{7,28} = 12.99$, $P < 0.001$; Fig. 3A), but only trended towards significance for melanized feathers (time by treatment interaction, $F_{7,28} = 12.99$, $P = 0.052$; Fig. 3B).

We did not detect a difference between inoculated melanized and unmelanized feathers (time by pigment by treatment interaction, $F_{7,56} = 1.10$, $P = 0.376$). Based on the criterion for the appearance of degradation discussed above, unmelanized feathers showed clear signs of degradation at 240 h (Fig. 3A), while there was no clear sign of when degradation began for melanized feathers within the time frame of our experiment (Fig. 3B).

Bacteria reached higher densities on unmelanized feathers compared to melanized feathers (time by pigment interaction, $F_{5,20} = 4.92$, $P = 0.001$; Fig. 4A). Unmelanized inoculated feathers lost more mass than unmelanized controls (time by treatment interaction, $F_{1,4} = 23.00$, $P = 0.009$) and either inoculated or control group melanized feathers (time by pigment by treatment interaction, $F_{1,8} = 19.61$, $P = 0.002$; Fig. 4B). There was no difference in mass loss between control and inoculated melanized feathers (time by treatment interaction, $F_{1,4} = 0.69$, $P = 0.451$; Fig. 4B).

Discussion

Resistance of melanized feathers to bacterial degradation

Irrespective of the feather sterilization method or bacterial activity metric used, unmelanized feathers degraded more
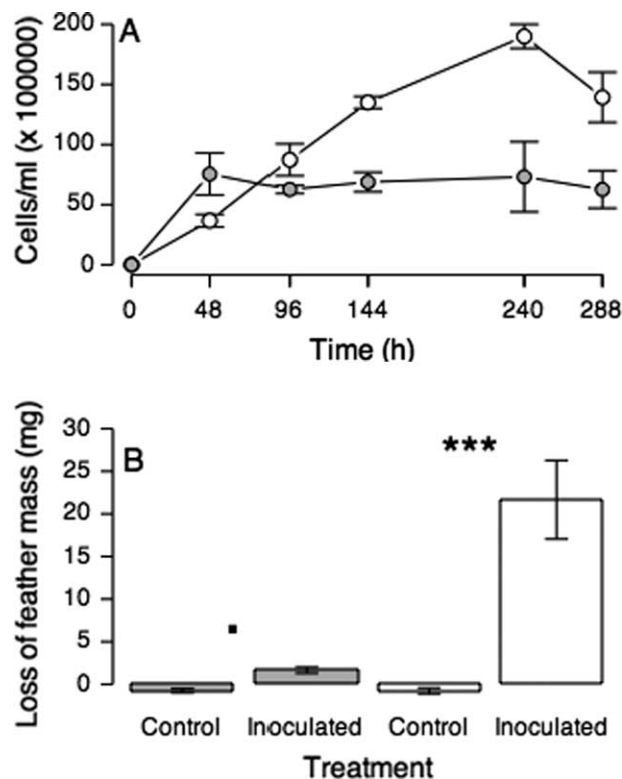

Figure 2. (A) Bacterial growth on domesticated goose feathers sterilized by autoclaving. *White circles*, growth on unmelanized feathers; *grey circles*, growth on melanized feathers. (B) Loss of feather mass of domestic goose feathers sterilized by autoclaving. *White bars*, unmelanized feathers; *grey bars*, melanized feathers. Bars in both (A) and (B) represent one standard error. (.) $0.10 > P > 0.05$, (***) $P < 0.001$.
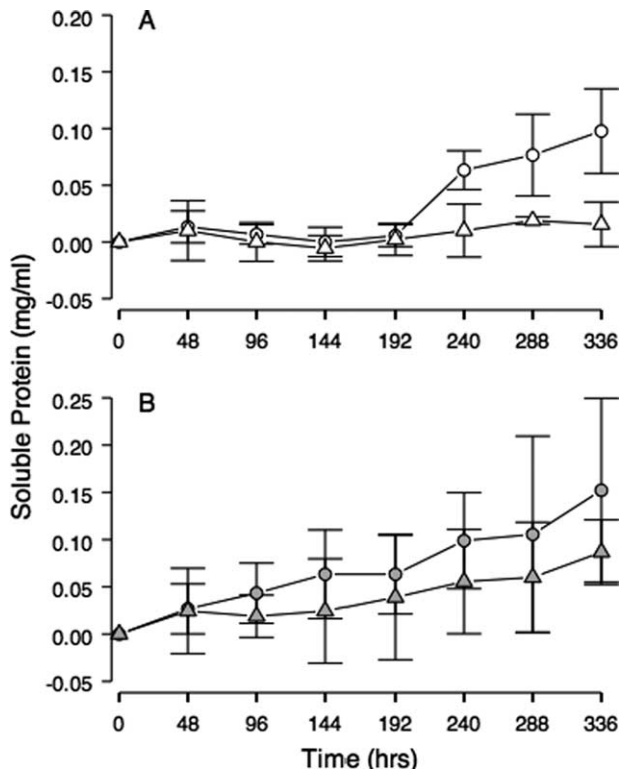
Figure 3. Mean soluble protein content of media surrounding domestic goose feathers sterilized with ethylene oxide gas and inoculated with *B. licheniformis*. (A) Unmelanized feathers. (B) Melanized feathers. *Circles*, feathers inoculated with *B. licheniformis*; *triangles*, uninoculated controls. Bars indicate 95% confidence intervals.

Figure 4. (A) Bacterial growth on domesticated goose feathers sterilized with ethylene oxide gas. *White circles*, growth on unmelanized feathers; *grey circles*, growth on melanized feathers. (B) Loss of feather mass of domestic goose feathers sterilized with ethylene oxide gas. *White bars*, unmelanized feathers; *grey bars*, melanized feathers. Bars in both (A) and (B) represent one standard error. (NS) Not Significant, (***) P <0.001.

readily than melanized feathers. In both trials, *B. licheniformis* grew to higher densities, removed more feather mass, and showed signs of degradation activity earlier on unmelanized relative to melanized feathers. These results support the conclusion of Goldstein et al. (2004) but contradict those of Grande et al. (2004). Melanized feathers are more resistant than unmelanized feathers to the degrading action of *B. licheniformis*. Hence, our study helps to resolve a current controversy in the literature and firmly indicates that melanins can render a feather more resistant to FDB.

Affects of sterilization on bacterial feather degradation

We show evidence that feather sterilization method can influence bacterial activity on feathers. Based on soluble protein content in feather solution, unmelanized feathers showed initial signs of degradation 48 hours earlier in the autoclave compared to the ethylene oxide trial (compare Fig. 1A and 3A). Melanized feathers showed initial signs of degradation at 240 hours in the autoclaved trial, but this never occurred in the ethylene oxide trial (compare Fig. 1B and 3B). Interestingly, in the autoclaved trial, bacterial populations crashed on unmelanized feathers (Fig. 2A), but this crash was not seen in the ethylene oxide trial (Fig. 4A). Furthermore, inoculated melanized feathers trended to-
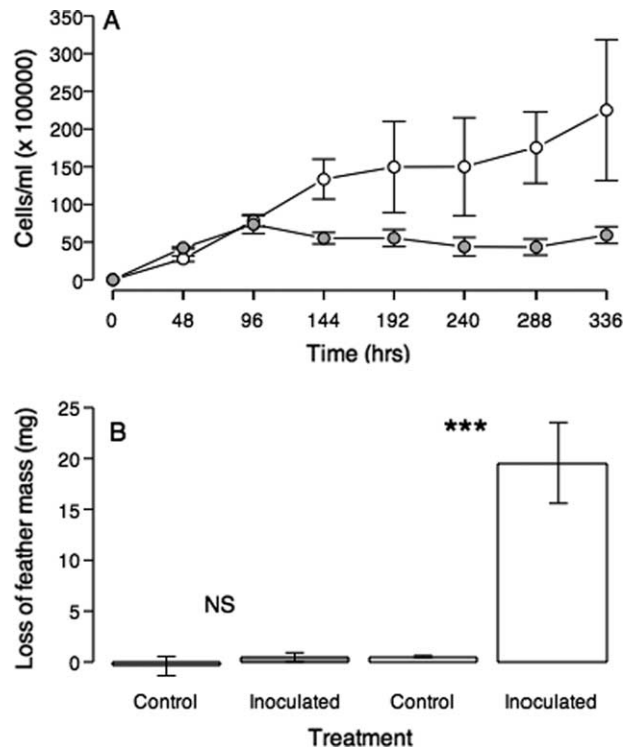
wards losing more mass (P =0.053) than control melanized feathers in the autoclave trial (Fig. 2B), but there was no difference in mass loss between these groups (P =0.451) in the ethylene oxide trial (Fig. 4B). These effects did not influence the qualitative interpretation of our data. In both the autoclave and ethylene oxide trials, unmelanized feathers were clearly more susceptible to bacterial degradation than melanized feathers. This is likely because the difference in degradation rates between these two feather types is large. However, if differences in degradation rate between two feather types is more subtle, the influence of feather sterilization method on bacterial activity could qualitatively influence the interpretation of results. We recommend that future studies comparing bacterial degradation rates of different feather types avoid autoclaving and employ feather sterilization techniques unlikely to influence feather structure, such as ethylene oxide gas (this study) or γ-rays (Shawkey et al. 2007).

We also found evidence that incubation conditions can differentially affect different feather types. Soluble protein content around control melanized feathers increased steadily over time in both trials (Fig. 1B and 3B), but did not occur with unmelanized controls (Fig. 1A and 3B). Thus, we strongly recommend that controls be prepared for all feather types, as the influence of FDB on feather condition must be measured relative to uninoculated feathers with the same properties.

Implications

The mechanism(s) of FDB resistance of melanized feathers is unknown (Goldstein et al. 2004). Goldstein et al. (2004) suggest that the incorporation of melanin granules into a feathers' keratin matrix may force keratin rods into close proximity with one another, thus catalyzing the production of more disulfide bonds between adjacent keratin molecules. Intermolecular disulfide bonds must be broken for keratins to become nutritionally available to FDB, and FDB produce specific enzymes and chemicals to reduce these bonds (Gupta and Ramnani 2006). More disulfide bonds could impede feather degradation. However, there is no evidence that melanins facilitate disulfide bonding among keratin molecules. At present, not enough is known about the biochemistry of melanized versus unmelanized feathers to determine if this is a reasonable hypothesis.

Melanins could also inhibit FDB by binding to bacterial proteolytic keratinases that hydrolyze keratins, a model for which there is experimental support. Melanized fungal mycelia resist microbial enzymatic digestion and, to test the mechanism of enzymatic resistance, Kuo and Alexander (1967) measured the efficiency of several microbial enzymes in the presence of melanin. Melanins reduced the efficiency of all enzymes, one of which was a protease of the bacterium *Bacillus subtilis* (Suh and Lee 2001), a FDB closely related to *B. licheniformis*. The activity of the *B. subtilis* protease was reduced by 49% to 69% in the presence of various concentrations of melanin and melanin itself was found to be resistant to microbial degradation (Kuo and Alexander 1967). Thus, it seems reasonable to suggest that one way in which melanins protect feathers is by binding to and inactivating keratinases.

The next step is to determine whether or not the resistance of melanized feathers to FDB is relevant to plumage under natural conditions. Burtt and Ichida (2004) began to address this question by comparing the prevalence (number of individuals infected) and feather-degradation rates of *B. licheniformis* from light and dark subspecies of song sparrow (*Melospiza melodia fallax* and *Melospiza melodia morphna*, respectively). The darker birds were assumed to have higher melanin concentrations in their feathers, although changes in the relative quantities of eumelanin and phaeomelanin could also play a role (McGraw 2006). There was no difference in the prevalence of FDB between the populations, but the data indicated FDB on the darker sparrow population degrade unmelanized chicken feathers more quickly than FDB isolated from the lighter sparrow population (Burtt and Ichida 2004). The authors interpret this as evidence that more damaging bacteria are selecting for more melanin deposition in feathers. However, they quantified bacterial degradation subjectively in a manner similar to Grande et al. (2004); no protein measurements, bacterial counts, or measurements of feathers-mass change were made. They also note that autoclaving the chicken feathers before inoculation damaged the feathers, and to different degrees in different flasks, which was not accounted for in their analysis. Furthermore, it is unclear how FDB degradation of unmelanized chicken feathers relates to the degradation of melanized sparrow feathers. If feather melanization puts selective pressure on FDB, then FDB should adapt to degrade feathers more readily in the presence of melanin, which, depending on the mechanism of melanic inhibition, would not necessarily influence the rate at which bacteria degrade unmelanized feathers. Roulin (2007) found that the rate of preening in barn owl *Tyto alba* nestlings decreased with plumage melanization. Preen oil can inhibit the growth of FDB (Shawkey et al. 2003) and the physical act of preening may dislodge or damage bacteria (Clayton 1999). If this is the case, then more melanized individuals may not need to preen as regularly to combat FDB. At present, well-controlled field experiments are needed to test the hypothesis that melanized feathers resist degradation by FDB. However, our series of laboratory tests, and those by Goldstein et al. (2004), indicate that melanin deposition can make feathers relatively more resistant to degradation by FDB. Therefore, it is possible that FDB can impose selection pressures on the evolution of avian feather colouration and the physical abilities of feathers to resist abrasion and damage.

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